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### Hypoxia, AMPK activation and uterine artery vasoreactivity

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**Title:** Hypoxia, AMPK activation and uterine artery vasoreactivity

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**Running head:** AMPK and uterine artery vasodilation

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## Key points summary

- Uterine artery vasodilatation is a key mechanism for increasing utero-placental blood flow and fetal nutrient supply.
- Since the pioneering work of Joseph Barcroft, the natural laboratory of high altitude has been used to study the mechanisms regulating uterine artery blood supply and fetal growth.
- Genes near the metabolic sensor, adenosine monophosphate-activated protein kinase (AMPK), have been implicated in genetic protection from high altitude-associated fetal growth restriction.
- We show that AMPK is present in utero-placental tissues, has vasodilator effects in murine uterine arteries, and that exposure to chronic hypoxia sufficient to decrease fetal growth increases the vasodilator actions of AMPK in opposing phenylephrine-induced vasoconstriction.
- These results point to AMPK as being a key link between maternal vascular responses to pregnancy and fetal growth. Manipulation of AMPK may be a novel mechanism for developing new therapies in pregnancies complicated by chronic hypoxia.

## Abstract

Genes near *PRKAA1* (adenosine monophosphate-activated protein kinase [AMPK]  $\alpha$ -1) have been implicated in the greater uterine artery (UtA) blood flow and relative protection from fetal growth restriction seen in altitude-adapted, Andean populations. AMPK activation vasodilates multiple vessels but whether AMPK is present in UtA or placental tissue and influences UtA vasoreactivity during normal or hypoxic pregnancy remains unknown. We studied isolated UtA and placenta from near-term C57BL/6J mice housed in normoxia (n=8) or hypoxia (10% F<sub>i</sub>O<sub>2</sub>, n=7-9) from day 14-19, and placentas from non-labouring sea level (n=3) or 3100 m (n=3) women. Hypoxia increased AMPK immunostaining in near-term murine UtA and placental tissue. RT-PCR products for AMPK  $\alpha$ -1 and  $\alpha$ -2 isoforms and LKB1 (the upstream kinase activating AMPK) were present in murine and human placenta, and hypoxia increased LKB1, AMPK  $\alpha$ -1 and  $\alpha$ -2 expression in the high- compared with low-altitude human placentas. Pharmacological AMPK activation by A769662 caused phenylephrine pre-constricted UtA from normoxic or hypoxic pregnant mice to dilate and this dilatation was partially reversed by the NOS inhibitor L-NAME. Hypoxic pregnancy sufficient to restrict fetal growth markedly augmented the UtA vasodilator effect of AMPK activation in opposition to PE constriction as the result of both NO-dependent and NO-independent mechanisms. We concluded that AMPK is activated during hypoxic pregnancy and that AMPK activation vasodilates the UtA, especially in hypoxic pregnancy. AMPK activation may be playing an adaptive role by limiting cellular energy depletion and helping to maintain utero-placental blood flow in hypoxic pregnancy.

**Keywords:** fetal growth restriction, high-altitude adaptation, nitric oxide, utero-placental blood flow, phenylephrine

**Abbreviations:** AICAR, 5-amino-1-beta-D-ribofuranosyl-imadazole-4-carboxamide; AMPK, AMP-activated protein kinase; AUC, area under the curve; BK<sub>CA</sub>, large-conductance potassium channel; BPD, biparietal diameter; DAB, 3, 3'-diaminobenzidine; JZ, junctional zone; KCl, potassium chloride; LKB1, liver kinase B1, LZ, labyrinthine zone; mTOR, mechanistic target of rapamycin; NO, nitric oxide; NOS, nitric oxide synthase; PE, phenylephrine; PRKAA1, the gene coding for AMPK alpha-1 isoform; THR, threonine; UtA, uterine artery.

## Introduction

Pioneering studies of Joseph Barcroft and his students drew attention to the value of high altitude as a natural laboratory for understanding the mechanisms by which intrauterine hypoxia reduces fetal growth (Barcroft, 1933; Barron *et al.*, 1964). A key determinant of fetal growth is the pregnancy rise in uterine artery (UtA) blood flow, which is due, in turn, to profound changes in multiple physiological systems (Gant & Worley, 1989). Among the greatest are the structural remodelling and alterations in vasoreactivity of the uterine vasculature that result in approximately 20% of the maternal cardiac output being directed to the utero-placental circulation by term (Osol & Moore, 2014). Isolated vessel studies in experimental animals show that chronic hypoxia is associated with a decreased vasodilator response to flow and to pharmacological agonists in the main UtA and downstream vessels (White *et al.*, 2000; Mateev *et al.*, 2003; Xiao *et al.*, 2010), which may, in turn, contribute to the reduced UtA blood flow and altitude-associated fetal growth restriction seen in high-altitude newcomers (Zamudio *et al.*, 1995; Julian *et al.*, 2008). Multigenerational highland residents (Andeans and Tibetans) are relatively protected from altitude-associated reductions in fetal growth and have a greater pregnancy rise in UtA blood flow compared with altitude newcomers (Moore *et al.*, 1998; Giussani *et al.*, 2001;

Moore *et al.*, 2001; Chen *et al.*, 2002; Julian *et al.*, 2007; Julian *et al.*, 2009; Soria *et al.*, 2013). Such protection appears due, in part, to genetic factors given that it is proportional to the degree of highland ancestry and is not the result of the woman's own duration of residence at high altitude (Bennett *et al.*, 2008; Julian *et al.*, 2011; Soria *et al.*, 2013).

Identifying the genes and signalling pathways involved in protecting native highlanders presents a novel means for addressing the mechanisms by which intrauterine hypoxia influences fetal growth. Several gene regions have been acted upon by natural selection in long-resident populations (Bigham *et al.*, 2009; Beall *et al.*, 2010; Bigham *et al.*, 2010; Simonson *et al.*, 2010; Yi *et al.*, 2010; Alkorta-Aranburu *et al.*, 2012). In Andeans prominent among these are single nucleotide polymorphisms (SNPs) near *PRKAA1* (*adenosine monophosphate-activated protein kinase [AMPK] alpha-1*) (Bigham *et al.*, 2009; Bigham *et al.*, 2010). Further, the *AMPK alpha-1* variants more common in Andeans are positively associated with infant birth weight as well as with key determinants of fetal growth, namely the pregnancy-associated increase in UtA diameter and the expression patterns of genes in metabolic pathways proposed to play a role in altitude-associated fetal growth restriction (Yung *et al.*, 2012; Bigham *et al.*, 2014).

AMPK is a ubiquitously expressed enzyme in eukaryotes, that is stimulated by stresses that deplete cellular ATP and thus serves as a metabolic sensor for matching tissue energy demand with supply (Hardie *et al.*, 2012). It is comprised of three subunits (alpha, beta, gamma), each of which has multiple isoforms whose expression levels vary by tissue type (Viollet *et al.*, 2010). The alpha-1 and alpha-2 isoforms are the catalytic subunits and contain the Thr-172 site where AMPK is activated more than 100-fold via phosphorylation by LKB1 and other upstream

kinases (Evans *et al.*, 2009; Viollet *et al.*, 2009). In skeletal muscle AMPK activation promotes glucose uptake and mitochondrial biogenesis, and decreases energy demand by inhibiting the mechanistic target of rapamycin (mTOR) and switching on various catabolic enzymes (Hardie, 2011). More recently AMPK activation has been recognized to have vascular effects, acting to stimulate endothelial nitric oxide (NO) production (Wang & Proud, 2006) as well as to regulate smooth muscle function directly (Goirand *et al.*, 2007). Underscoring its importance under conditions of hypoxia, AMPK activation has been implicated in the aetiology of early-onset pre-eclampsia (Yung *et al.*, 2014a) cardiorespiratory responses to hypoxia (Evans, 2006), and hypoxic pulmonary vasoconstriction (Evans *et al.*, 2005).

It is unknown whether AMPK activation influences UtA vasoreactivity during pregnancy and, if so, whether such effects are altered by exposure to hypoxia. We used a broad-ranging approach to address such questions in which human as well as experimental-animal tissues were studied using multiple methods. Specifically, we hypothesized that AMPK is present in UtA and placental tissue and that its expression was increased during pregnancy by exposure to hypoxia as detected using immunohistochemistry in mice and quantitative RT-PCR in previously collected human tissues. We further hypothesized that AMPK activation prompted UtA vasodilation and/or altered vasoconstrictor sensitivity to phenylephrine (PE) in vessels isolated from near-term mice, and that such effects were altered by exposure to hypoxia. To test these hypotheses we used the pharmacological AMPK agonist A769662 and compared UtA vasodilator and vasoconstrictor responses in vessels isolated from the normoxic vs. hypoxic animals. Finally, we treated vessels with the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) to determine the contribution of NO-dependent and

-independent mechanisms to the effects of AMPK activation observed. We considered that such studies would improve our understanding of the mechanisms regulating maternal vascular responses to pregnancy and fetal growth under conditions of chronic hypoxia.

## Methods

Ethical Approval. All mouse experiments were carried out using procedures consistent with the UK Animals Scientific Procedures Act 1986 and approved by the Local Ethics Review Committee of the University of Cambridge. Placentas were collected from human subjects who provided written informed consent to procedures approved by the University of Colorado Multiple Institutional Review Board (COMIRB, Aurora, CO, USA), the University College Hospital London (London, UK), and the Cambridge Local Research Ethics Committee.

Samples and Protocols. Female C57BL/6J mice were placed with males overnight. The presence of a copulatory plug the following morning was taken to indicate day one of pregnancy. Pregnant mice (n=31) were housed in groups of two or three in rooms with 21% O<sub>2</sub>, 12 hr light-dark cycles and controlled temperature (21°C) and humidity (60%). They had *ad libitum* access to food (Rat and Mouse No. 3 Breeding, Special Diet Services, Witham, UK). At day 14 of pregnancy (term is ~ 21 days), animals were randomly assigned to normoxic or hypoxic (10% O<sub>2</sub>) treatment groups. Hypoxia was achieved by placing the animals in a chamber containing a PVC isolator (PFI plastics Ltd) and a N<sub>2</sub> generator (N2MID60, Domnick Hunter Ltd, UK) so as to control the percent O<sub>2</sub> within the chamber, without changing the CO<sub>2</sub> levels, by altering the inflows of air and N<sub>2</sub>. O<sub>2</sub> levels were monitored using an O<sub>2</sub> analyser (ICA, UK) and CO<sub>2</sub> levels by a portable CO<sub>2</sub> analyser that was



calibrated daily (The Electronic Workshop, Department of Physiology, Development and Neuroscience, University of Cambridge). Normoxic animals were housed in the same room that contained the chambers. Maternal weight, food and water intake were monitored daily. This was achieved in the hypoxic group via a sealed transfer box that could be opened briefly without altering O<sub>2</sub> levels within the main chamber.

On day 19 of pregnancy mice were euthanized by cervical dislocation. The uterus was dissected immediately, the numbers of viable fetuses and fetal reabsorptions counted, fetal and placental weights recorded, and fetal biometry taken. Both UtA were removed, placed in ice cold PBS, and processed immediately (<5 min) for either myography or immunohistochemistry. In approximately half the normoxic and hypoxic animals, one placenta whose weight was closest to the litter mean was prepared for immunohistochemistry.

Samples from human placentas were obtained from term, non-labouring women residing either at sea level (n=3) or high altitude (3100 m, n=3) as described previously (Yung *et al.*, 2012).

Immunohistochemistry. The murine uterine vessels were placed in ice cold PBS, cleared of adipose tissues and the uterine horn using a dissecting microscope (Leica, Germany), and fixed in 4% paraformaldehyde. The main UtA together with its 1<sup>st</sup> and 2<sup>nd</sup> order branches was divided into 2-3 longitudinal segments, embedded in a single paraffin block, and sectioned. Whole placentas were washed with PBS, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Paraffin-embedded sections were de-waxed, washed, prepared for antigen retrieval using 0.01 M citric buffer (pH 6.0), and incubated overnight using either primary antibody (phospho-AMPK $\alpha$  [Thr172] [40H9] rabbit) from Cell Signalling Technology (Hitchin, UK) diluted in 5% GS/HS in TBS or vehicle alone. The following day, the secondary antibody

(anti-rabbit IgG, diluted 1:200 in 5% GS/HS) was applied, incubated for 1 hr, developed for staining using DAB and counterstained with hematoxylin. The slides were scanned using a Nanozoomer (Hamamatsu Photonics, Welwyn Garden City, UK), saved as high-resolution files, and images quantified using IPLAb software (v6.0, Scanalytics, Fairfax, VA). DAB-positive areas were selected and segmented in a region of interest based on a selective distribution of saturation and hue values that matched the color of the DAB reactive product and the pattern of staining of the image. Segmented overlays representing the selected pixels of DAB staining were selected in individual UtA and the labyrinthine and junctional zones (separately), quantified and expressed as the percent area of positive DAB staining per area of tissue examined.

Quantitative RT-PCR. RNA from mouse (n=3) or human (n=6) placental tissue was extracted using the miRNeasy Mini Kit from Qiagen (Manchester, UK) following the manufacturer's guidelines, and the concentration determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). cDNA synthesis was carried out using the Transcriptor High Fidelity cDNA synthesis Kit (Roche, UK) following manufacturer instructions. For qPCR analysis, 2.5 µl of cDNA in RNase free water was made up to 25 µl with FastStart Universal SYBR Green Master (ROX, 12.5 µl, Roche), Ultra Pure Water (8 µl, SIGMA, UK) and forward and reverse primers for LKB1, AMPK alpha-1 and alpha-2 (Qiagen, UK). The sample was then centrifuged and 25 µl added to a MicroAmp<sup>TM</sup> Fast Optical 96-Well Reaction Plate (Greiner Bio-One, Stonehouse UK), the reaction plate sealed with an optical adhesive cover (Applied Biosystems, Warrington UK) and the plate centrifuged. The reaction was then run on a sequence detection system (Applied Biosystems) using AmpliTaq Fast DNA Polymerase with a 2 min initial step at 50°C

followed by a 10 min step at 95°C, a 15 sec step at 95°C (repeated 40 times) followed by a dissociation stage with a 15 sec step at 95°C, and followed by a 20 sec at 60°C and a 15 sec step at 95°C. Negative controls included tissue aspirants for which no reverse transcriptase was added, and aspiration of extracellular medium and PCR controls. None of the controls produced any detectable amplicon, ruling out genomic or other contamination.

Myography. The murine uterine vasculature was dissected and pinned out in a dish of ice-cold Krebs solution (in mmol/L, all reagents from Sigma UK unless specified otherwise: NaCl 118.5, NaHCO<sub>3</sub> 25, KCl 4.75, MgSO<sub>4</sub>·7H<sub>2</sub>O 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5, D-glucose 11.1). Using a bifocal dissecting microscope (Brunel Microscopes Ltd., UK), first-order 2 mm in length UtA segments were dissected and cleared of any connective or adipose tissue. The vessel segments were then mounted in a four-chamber, small-vessel wire myograph (Multi Wire Myograph System 620M, DMT, Denmark) by threading two wires 40 µm in diameter through the vessel lumen and attaching one to a pressure transducer and the other to a micrometer as previously described (Pulgar *et al.*, 2011; Giussani *et al.*, 2012). The chamber was continually gassed with 5% CO<sub>2</sub> and 95% O<sub>2</sub> to maintain a physiological pH, and gradually heated to 37°C. Throughout the investigation, the Krebs solution was refreshed every 20 min, and the vessels allowed to re-equilibrate for at least 20 min between experiments.

UtA were normalised to 0.9 of L<sub>13.3 kPa</sub>, and allowed to equilibrate for 20 min. Following normalisation a 'wake up' protocol was performed consisting of exposure of the vessels to a high concentration of potassium (KCl, 125 mmol/L). Vessel viability was verified by a positive constrictor response to phenylephrine (PE, 1x10<sup>-4</sup> M) and a positive relaxant response to acetylcholine (ACh, 1x10<sup>-10</sup> M to 1x10<sup>-4</sup> M).

Two vessels from each animal were pre-constricted with a dose of PE determined to produce 70% of the maximal constriction to PE for each vessel. The sub-maximally pre-constricted vessels were then relaxed by addition of the AMPK agonist A769662 (Tocris, USA) dissolved in DMSO in increasing concentrations ( $1 \times 10^{-6}$  M to  $1 \times 10^{-4}$  M) at 10-min intervals, and the results from the two vessels per animal averaged. We chose A769662 because, unlike 5-amino-1-beta-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) or metformin, it activates AMPK directly, and not other enzymes that are responsive to AMP levels (Goransson *et al.*, 2007). Another advantage is that A769662, unlike AICAR, is not taken up by the adenosine transporter and therefore does not lead to the accumulation of adenosine outside cells or necessitate the use of an adenosine antagonist (Goransson *et al.*, 2007). A third and fourth vessel segment from each animal was also tested using increasing doses of PE ( $1 \times 10^{-9}$  M –  $1 \times 10^{-4}$  M), with this then being repeated following 20 min incubation with A769662 ( $1 \times 10^{-4}$  M) and finally repeated a third time following incubation with A769662 ( $1 \times 10^{-4}$  M) plus the NOS inhibitor L-NAME ( $1 \times 10^{-5}$  M). The finding that the third curve was consistently larger than the second provided reassurance that the response of the vessels was not deteriorating over time. Studies were completed in vessels from a total of 17 animals.

Data and Statistical Analyses. The contractile response to PE was expressed as a percentage of the maximal constriction produced by KCl (%  $K_{max}$ ). The curves were fitted with the best-fit line for describing the response to a given agonist. The negative logarithm to base 10 of the dose at which vessels were 50% maximally-constricted to PE ( $EC_{50}$  or  $pD_2$ ) was used as an index of vascular sensitivity, and the area under the curve (AUC) was calculated to assess overall reactivity using Prism v6.0 (GraphPad Software, La Jolla CA). Single values were compared between

normoxic and hypoxic groups using unpaired t-tests. Differences in immunostaining or mRNA expression between normoxic and hypoxic groups were assessed using one-way ANOVA with Student Newman Keuls *post hoc* test. The effects of AMPK activation on PE-induced constriction were assessed using two-way ANOVA with Tukey *post hoc* test (Sigma Stat, Buckinghamshire, UK). Data are presented as the mean  $\pm$  standard error of the mean (S.E.M.). Significance was accepted when the two-tailed  $p < 0.05$  and reported as trends when  $0.05 < p < 0.10$ .

## Results

Maternal and fetal characteristics. Murine maternal body weights were similar at days 1 and 14, but reduced at day 19 in the hypoxic compared with normoxic groups (Table 1). Litter size was not reduced in the hypoxic group although there was a trend for a greater number of reabsorptions. Fetuses from hypoxic pregnancies weighed less but placental weights were similar to those seen in the normoxic group. Fetuses from the hypoxic compared with the normoxic group had smaller crown-rump lengths, biparietal diameters (BPD) and a greater BPD to body weight ratio, indicating asymmetric growth restriction (Table 1).

Placental and birth weights were similar for the non-labouring, sea-level or high-altitude women (Table 1), although birth weight was reduced at high altitude in the larger group of subjects from which these data were derived (Yung *et al.*, 2012)

Immunohistochemistry. The uterine vessels from normoxic mice demonstrated staining for phosphorylated (activated) AMPK in arteries of varying size. The hypoxic compared with normoxic animals had greater staining intensity with primary antibody (22.0% vs. 11.0% respectively,  $p < 0.0001$ ) whereas the staining intensity did not differ in the vessels from normoxic animals with and without

antibody ( $p=0.12$ ), indicating minimal AMPK expression (Figure 1, left hand panels). Phosphorylated AMPK was also present in all regions of the mouse placenta (Figure 1, right hand panels). Whereas staining intensity did not differ between the JZ and LZ of the placenta from normoxic animals ( $p=NS$ ), hypoxic exposure markedly increased LZ relative to JZ staining ( $p<0.0001$ ).

Quantitative RT-PCR. Results from quantitative RT-PCR assays showed that mRNA for LKB1, AMPK alpha-1, and AMPK alpha-2 (as a % of  $\beta$ -actin) were present in human and mouse placental tissue. Levels of RT-PCR products for LKB1 and both AMPK catalytic isoforms were greater in the tissues from the high-altitude compared with sea-level human placentas (Figure 2, top panel). In sea-level mouse placentas, AMPK alpha-2 levels were higher in the labyrinthine than junctional zones (Figure 2, bottom panel).

Myography. The internal circumference of the isolated UtA at the time of study was similar in the normoxic and hypoxic murine groups (Table 1). There was no difference in the maximal contraction to KCl or PE, the contractile sensitivity to PE as measured by the  $pD_2$  (Table 1), or the relaxation response to ACh ( $10^{-10}$  M to  $10^{-4}$  M,  $p=NS$ , data not shown).

A769662, an AMPK agonist, caused complete, concentration-dependent relaxation in the PE pre-constricted UtA and that relaxation was similar in the normoxic and hypoxic mice (Figure 3A). Inhibition of NOS by incubation with L-NAME reduced the relaxation response to A769662 by 30% but did not fully reverse it, indicating that 70% was NO-independent in both the normoxic and hypoxic groups (Figure 3B).

Incubation with the AMPK agonist A769662 reduced UtA contractile sensitivity to PE in both the normoxic and hypoxic mice as demonstrated by the rightward shifts in the contractile concentration-response curves (Figure 4A-B), with the change due to A769662 being greater in the hypoxic than normoxic groups (Figure 4C).

Treatment of the vessel with L-NAME increased contractile sensitivity to PE in vessels from normoxic or hypoxic mice (two-way ANOVA, both  $p < 0.05$ ), indicating that a portion of A769662's effect in opposing PE-induced vasoconstriction was likely due to increased NO production or activity. However, L-NAME treatment did not fully reverse A769662's effect, indicating that there was a NO-independent contribution as well. Moreover the NO-independent component was principally responsible for the greater vasodilator effect of AMPK activation seen in the vessels from hypoxic than normoxic animals (Figure 4D).

## Discussion

Our principal findings were that AMPK was present in uterine arteries from near-term mice as well as in mouse and human placental tissue, and that AMPK expression was increased by hypoxia as judged by immunohistochemistry and mRNA expression. Consistent with the possibility that the LKB1-AMPK signalling pathway influenced vascular reactivity, we found that pharmacological activation of AMPK caused PE-precontracted UtA from pregnant mice to relax due to both NO-dependent and NO-independent mechanisms. While the vasodilator effect of AMPK activation was similar in UtA isolated from normoxic or hypoxic mice, AMPK activation more markedly opposed PE-induced constriction in the UtA from the hypoxic than the normoxic mice and this was due to NO-dependent but largely NO-independent

mechanisms. We therefore concluded that AMPK activation dilates uterine arteries, particularly in response to PE-induced vasoconstriction, and that this latter effect is enhanced by hypoxic pregnancy.

AMPK activation in non-pregnant animals has chiefly been studied for its metabolic effects. Drugs such as metformin, which is widely prescribed for lowering glucose levels in individuals with type 2 diabetes, decrease hepatic glucose production by mildly inhibiting the mitochondrial respiratory chain complex I, which in turn activates AMPK (Viollet *et al.*, 2012). In skeletal muscle, AMPK activation promotes glucose uptake and mitochondrial biogenesis, and decreases energy demand by inhibiting the mechanistic target of rapamycin (mTOR) or by switching on the expression of various catabolic enzymes (Hardie, 2011). More recently it has been recognized that AMPK activation also has vascular effects. Vessels express AMPK alpha-1 and alpha-2 isoforms; alpha-1 predominates in endothelial cells and both are present in vascular smooth muscle with their relative predominance varying by tissue type (Evans *et al.*, 2005; Goirand *et al.*, 2007; Matsumoto *et al.*, 2008).

AMPK activation has been shown to improve cardiac function in a rat model of chronic heart failure (Wang *et al.*, 2011); to help initiate hypoxic pulmonary vasoconstriction (Evans *et al.*, 2005); and to augment acetylcholine-induced relaxation in pre-constricted isolated thoracic aorta, mesenteric or resistance-sized cremaster arteries (Goirand *et al.*, 2007; Ford & Rush, 2011). Importantly, the vascular effects of AMPK activation are not dependent on its metabolic effects (Bradley *et al.*, 2010) or, as is the case with AICAR, the accumulation of adenosine (Evans *et al.*, 2005; Goirand *et al.*, 2007; Bradley *et al.*, 2010) but rather are due to both NO-dependent and NO-independent mechanisms; namely, increased eNOS activity and NO production (Viollet *et al.*, 2010), decreased superoxide and



vasoconstrictor prostanoid production (Matsumoto *et al.*, 2008; Li *et al.*, 2010), and direct actions in vascular smooth muscle (Goirand *et al.*, 2007).

Our data showing AMPK immunostaining in normoxic mouse uterine vessels and placenta tissue, and mRNA in murine and human placenta for both AMPK alpha isoforms and the upstream kinase responsible for activating AMPK, LKB1, in mouse and human placenta were consistent with prior reports showing that AMPK is expressed in placental tissue (Yung *et al.*, 2012). This is, however, to the best of our knowledge the first report of its presence in murine uterine vessels during pregnancy. The UtA and other uterine vessels undergo profound changes during pregnancy, enlarging their diameters as the result of structural remodelling as well as increased vasodilator responses to flow, acetylcholine and other pharmacological agonists (reviewed in (Osol & Moore, 2014)). Such changes are attributable to effects of oestrogen and other pregnancy hormones serving to increase the production and/or activity of various vasodilators, including NO and large-conductance potassium channels (BK<sub>CA</sub>), rather than placentation *per se* since the changes begin before placentation is complete, occur even in ectopic pregnancy, and are present albeit to a lesser degree in pseudo-pregnant animals (Burchell, 1967; Rosenfeld *et al.*, 1996; van der Heijden *et al.*, 2005; Collins *et al.*, 2011; Hu *et al.*, 2011). The present report shows for the first time that uterine arteries are relaxed by AMPK activation, suggesting that activation of AMPK may be another mechanism contributing to pregnancy vasodilatation. The vasorelaxant effects of AMPK activation were due, in part, to increased NO production or activity as shown by the effect of NOS inhibition but the major portion of its vasorelaxant effect remained following NOS inhibition, indicating that additional NO-independent mechanisms are involved. One possibility is that AMPK activation reduced the production of

vasoconstrictor prostanoids, similar to what has been reported in mesenteric arteries following metformin treatment in a rat model of type 2 diabetes (Matsumoto *et al.*, 2008). Direct effects on vascular smooth muscle cells may also be involved via actions on the RhoA-Rho associated protein kinase (Rock) pathway (Gayard *et al.*, 2011) or via K<sup>+</sup> channels given that AMPK activation affects multiple K<sup>+</sup> channels (Andersen & Rasmussen, 2012), including the BK<sub>CA</sub> and the ATP-sensitive potassium channel (K<sub>ATP</sub>), previously implicated in uterine vascular responses to pregnancy and hypoxia (Zhu *et al.*, 2013). Thus further study is required in both non-pregnant and pregnant animals to determine whether pregnancy influences the effects of AMPK activation on UtA vasoreactivity, and the endothelial as well as vascular smooth muscle mechanisms involved.

Little is known about the effects of AMPK activation under conditions of hypoxia. Suggesting a beneficial role, Davidge and co-workers have shown that resveratrol, which works in part by activating AMPK (Hardie, 2011; Tennen *et al.*, 2012), administered to the mother under conditions of severe hypoxia dramatically improved fetal survival and increased placental relative to fetal weight but not fetal growth *per se* (Bourque *et al.*, 2012; Banek *et al.*, 2013). Resveratrol treatment also increased UtA blood flow velocity and fetal weight in a catechol-O-methyltransferase knockout mouse model of fetal growth restriction, but vasodilator or vasoconstrictor responses of isolated UtA were unaffected (Poudel *et al.*, 2013). Resveratrol has also been shown to augment UtA blood flow during pregnancy in nonhuman primates (Roberts *et al.*, 2014).

We found that hypoxia in late murine pregnancy sufficient to decrease fetal growth increased the intensity of AMPK immunostaining in the labyrinthine zone, which may be more sensitive to hypoxia given its rich blood supply in contrast to the relatively hypovascularized junctional zone (Dilworth & Sibley, 2013). AMPK alpha-2 mRNA expression was also greater in the labyrinthine than the junctional zone of the mouse placenta, suggesting that it may be the isoform involved in the greater immunostaining but differences in primer efficiency prevent comparison of LKB1, AMPK alpha-1 and alpha-2 expression levels. In humans, the levels of RT-PCR products for both AMPK alpha subunits and LKB1 were greater in the placentas from high- vs. low-altitude residents, suggesting higher basal AMPK activity and / or increased capacity to respond to metabolic stress. However, while these same placentas showed evidence of endoplasmic reticulum (ER) stress and mTOR inhibition, the ratio of P-AMPK to total AMPK was not consistently elevated in the small number (n=3) of non-labouring placentas available for study (Yung *et al.*, 2012). In pre-eclamptic women, the ratio of P-AMPK to total AMPK protein levels was inversely correlated with gestational age, being highest in those with the earliest onset of disease, although not higher overall in early- (<34 wk) vs. late ( $\geq 34$  wk) onset pre-eclamptic or normotensive women (Yung *et al.*, 2014b). Thus, further studies in larger numbers of placentas are required to determine if AMPK is activated at high vs. low altitude in placental tissue.

We were interested in the effect of AMPK activation on UtA vasoreactivity under conditions of hypoxia given our prior studies indicating that the gene region containing *AMPK alpha-1* has been acted upon by natural selection in Andean residents of high altitude and that the variants more common in Andeans were positively associated with birth weight, UtA diameter and alterations in the

expression of genes in the mTOR pathway previously implicated in altitude-associated fetal growth restriction (Bigham *et al.*, 2009; Yung *et al.*, 2012; Bigham *et al.*, 2014). The increased intensity of immunostaining in the mouse uterine arteries suggested that hypoxia increased AMPK abundance. However, since the antibody employed detects both alpha-1 and alpha-2 isoforms, we were not able to determine which isoforms were present in the uterine vessels.

Based on previous studies in pulmonary and uterine arteries, we expected that AMPK activation would have less vasodilator effect in the UtA isolated from the hypoxic vs. normoxic animals. In pulmonary arteries, AMPK activation under conditions of acute ( $\leq 60$  min) hypoxia prompted vasoconstriction through mobilization of myocyte sarcoplasmic reticulum calcium stores via ryanodine receptors and the release of an endothelium-derived constrictor (Evans, 2006). Inhibitory effects of AMPK activation on Bk<sub>CA</sub> channels have also been observed, and in a manner that is splice-variant specific (Wyatt *et al.*, 2007; Ross *et al.*, 2011). It is notable, therefore, that Bk<sub>CA</sub> channels are inhibited in resistance-sized ovine uterine vessels and, in turn, reverse the normal pregnancy reduction in myogenic tone (Hu *et al.*, 2012). Further, we have reported that hypoxia throughout pregnancy reduced the NO-dependent UtA vasodilator response to ACh and flow in isolated guinea pig UtA (White *et al.*, 2000; Mateev *et al.*, 2003). Therefore, our finding that pharmacological AMPK activation prompted similar concentration-dependent vasodilation in precontracted UtA from normoxic and hypoxic animals was somewhat surprising. Since maximal contraction to PE tended ( $p=0.07$ ) to be greater in the hypoxic than the normoxic group and in order to evaluate the relationship between AMPK activation and PE constriction more fully, we extended our study to examine the effect of AMPK activation on contractile sensitivity to PE. We found that

AMPK activation markedly reduced UtA contractile sensitivity to PE in the UtA from the hypoxic compared with normoxic animals as demonstrated by a rightward shift in the contractile-response curves. The shift was greater in the vessels from the hypoxic compared to normoxic animals, and was partly reversed by treatment with the NOS inhibitor L-NAME, suggesting a role for NO in opposing PE-induced of constriction. While we cannot rule out the possibility that hypoxic exposure increased NO sensitivity, this appeared unlikely given that the relaxation in response to ACh or A769662 was not greater in the UtA from the hypoxic compared to normoxic animals. In addition, even though the NO component was larger in the hypoxic group, the major portion of the greater reduction in contractile sensitivity to PE was NO-independent. We therefore concluded that greater AMPK activation under conditions of hypoxic pregnancy opposes PE-induced vasoconstriction, thus perhaps serving as a compensatory mechanism for maintaining uterine artery blood flow. Further studies are required for determining the mechanisms by which AMPK activation opposes PE-induced vasoconstriction and whether similar effects of AMPK activation are observed when hypoxia is present throughout gestation such as is the case in residents of high altitude.

The strengths of our study were its broad-ranging design in which human as well as murine tissues were examined following exposure to normoxia and either shorter- or longer-term hypoxia. Further, multiple methods were used to evaluate the effects of AMPK activation and hypoxia; namely, immunohistochemistry, quantitative RT-PCR and myography. Our results, however, were limited by use of a single AMPK activator. While A769662, unlike other agonists, activates AMPK directly (Goransson *et al.*, 2007), it too has indirect effects by, for example, inhibiting the sodium pump (Benziane *et al.*, 2009). While we used only one agonist, our results

were similar to those obtained using metformin, AICAR or exercise to activate AMPK, and thus suggested that the responses observed were the result of AMPK activation and not some other effect of the agonist employed (Goirand *et al.*, 2007; Ford & Rush, 2011; Wang *et al.*, 2011; Kroller-Schon *et al.*, 2012) but additional studies using other agonists are required to confirm the mechanisms of A769662 action. Further studies are also needed using western blot to document AMPK activation, knock-out mice for clarifying the roles of the AMPK alpha-1 and AMPK alpha-2 isoforms, and pair-fed animals to control for the lesser maternal weight gain seen during the period of hypoxic exposure in the present report. The possible effect of duration of hypoxic exposure also requires evaluation since the late-pregnancy onset employed here does not parallel the circumstances of residence at high altitude where hypoxia is present throughout gestation but, on the other hand, it does likely resemble that occurring with pre-eclampsia.

In summary, AMPK is present in maternal uterine vessels as well as in placental tissues, and its abundance increased by short- as well as longer-term hypoxia as indicated by greater immunostaining in uterine vessels from pregnant mice and increased expression of both catalytic AMPK subunits in murine and human placenta. This is, to our knowledge, the first report that pharmacological AMPK activation prompts vasodilatation in UtA via both NO-dependent and NO-independent mechanisms, and that exposure to chronic hypoxia sufficient to restrict fetal growth markedly augmented the ability of AMPK activation to oppose PE-induced vasoconstriction. Together with prior reports that genetic variation near AMPK is related to increased UtA blood flow and maintenance of fetal growth in high-altitude adapted Andeans, we speculate that AMPK activation may be helping to maintain utero-placental blood flow and thereby limit cellular energy depletion. In

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addition to its role as a metabolic sensor, AMPK may be a key link between maternal metabolic and cardiovascular responses to pregnancy and the regulation of fetal growth.

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### **Author contributions**

All authors contributed to the conception and design or analysis and interpretation of data; the drafting the article or revising it critically for important intellectual content; and provided final approval of the version to be published. In particular, the idea for the study originated with LGM and GJB, ALF and ANS-P provided the animals, JSH and ANS-P collected the tissue, KLS and DAG carried out the myography studies, ADM and AME conducted the quantitative RT-PCR assays, and HWY and LGM performed the immunohistochemistry. Studies were conducted at the Centre for Trophoblast Research and Department of Physiology, Development and Neuroscience at the University of Cambridge, Cambridge UK with the quantitative RT-PCR assays being carried out at the Centre of Integrative Physiology at the University of Edinburgh, Edinburgh UK.

**Figure 1.** Phosphorylated AMPK (brown staining) is present in uterine arteries (UtA) and placentas from normoxic mice and increased by hypoxia in the UtA and the placental labyrinthine (LZ) but not junctional (JZ) or the decidua (DEC) zones. For the placenta specimens, panels A and B are with and without AMPK antibody, respectively, at 5x and panels C and D are with AMPK antibody at 20x magnification.

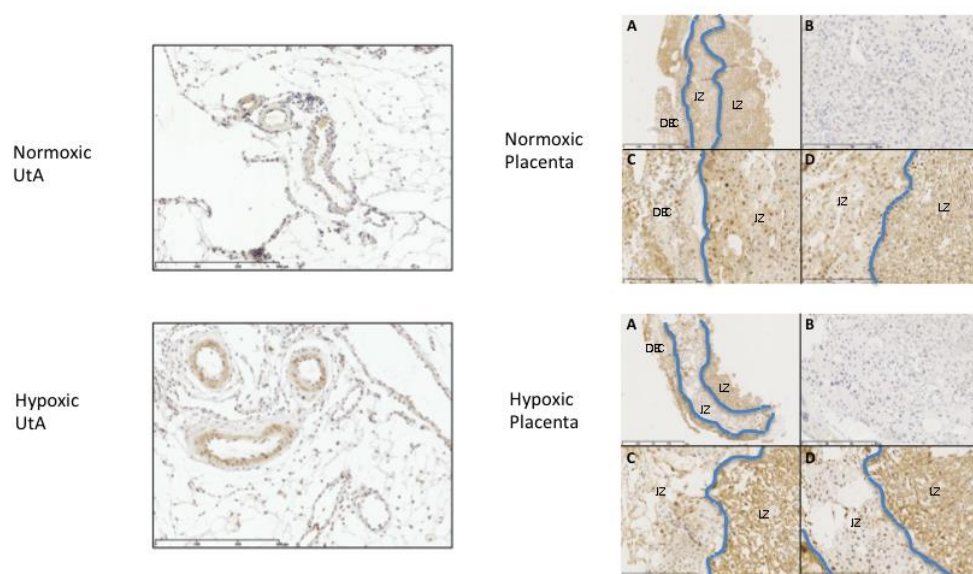


Figure 1

**Figure 2.** Upper Panel: mRNA for both AMPK alpha subunits and LKB1 are present at higher levels in human placentas from 3100 m vs. sea level. Lower Panel: Murine placentas also contain both AMPK alpha subunits and LKB1 at sea level, with expression levels for the alpha-2 subunit being greater in the labyrinthine than junctional zone. \*=p<0.05, \*\*=p<0.01, \*\*\*\*=p<0.0001. Data are mean  $\pm$  sem.

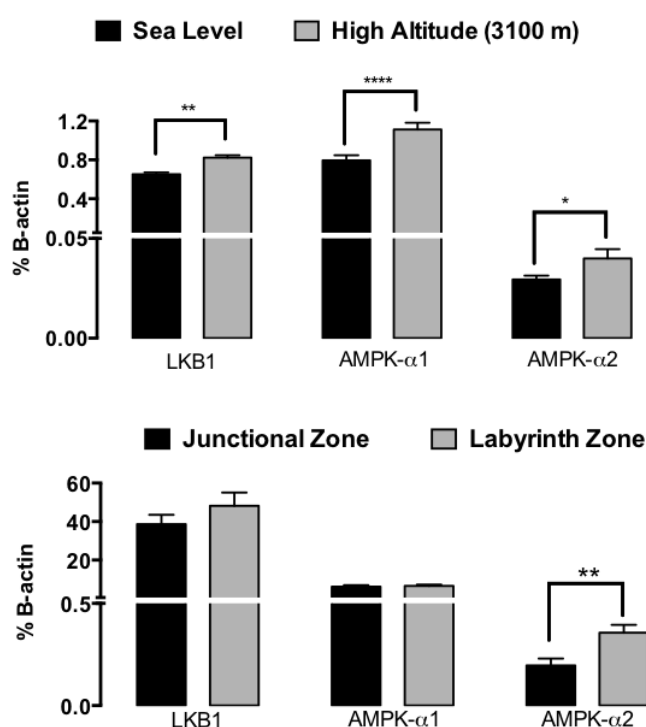


Figure 2



**Figure 3.** Panel A: Uterine arteries pre-constricted with a submaximal dose of PE from normoxic (n=5) and hypoxic (n=7) pregnant (day 19) mice relax in response to increasing concentrations of the AMPK agonist, A769662. Panel B: The area under the curve (AUC), representing nitric oxide (NO)-dependent and -independent components did not differ in the normoxic and hypoxic groups. Data are mean  $\pm$  sem.

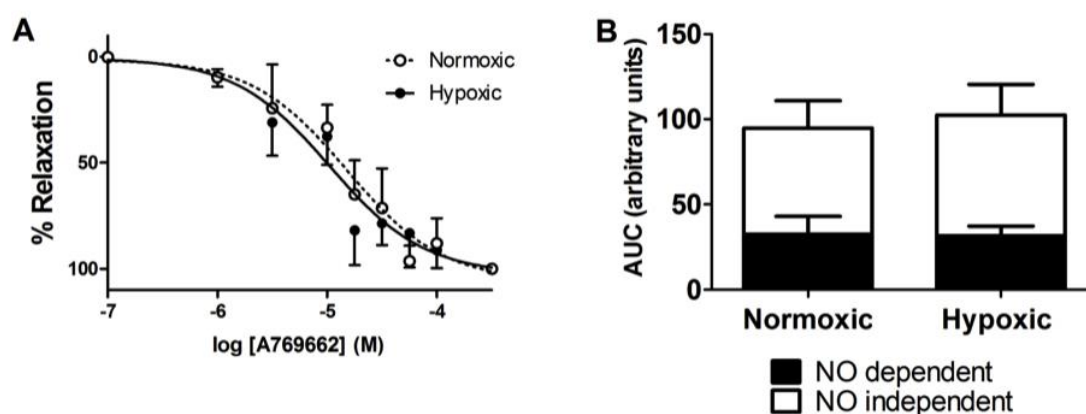


Figure 3

**Figure 4.** Incubation with A769662 ( $1 \times 10^{-4}$  M, open circles, dotted lines) decreased contractile sensitivity to phenylephrine (PE) in UtA from both normoxic (panel A, n=8) and hypoxic (panel B, n=6-7) pregnant mice compared to values obtained prior to incubation with A769662 (open circles, solid lines). This decrease was significant in both groups when expressed as the area under the curve (AUC) (panel C,  $p < 0.05$ , two way ANOVA and *post-hoc* Tukey test). The AUC, representing the change in sensitivity to PE following incubation with A769662, was significantly greater in the hypoxic compared to the normoxic animals (panel C,  $p < 0.05$ , unpaired t-test). Treatment with L-NAME ( $1 \times 10^{-5}$  M, black boxes and dashed lines) decreased the contractile response to PE in A769662-treated vessels from normoxic (A) and hypoxic (B) pregnant mice, indicating a significant NO-dependent component (both  $p < 0.05$ ), but did not fully restore the contractile response. The contributions of both nitric oxide (NO)-dependent and -independent mechanisms as well as the total relaxation response were greater in the UtA from hypoxic compared with normoxic animals (panel D,  $p < 0.05$ , unpaired t-test).

\* =  $p < 0.05$ . Data are mean  $\pm$  sem.

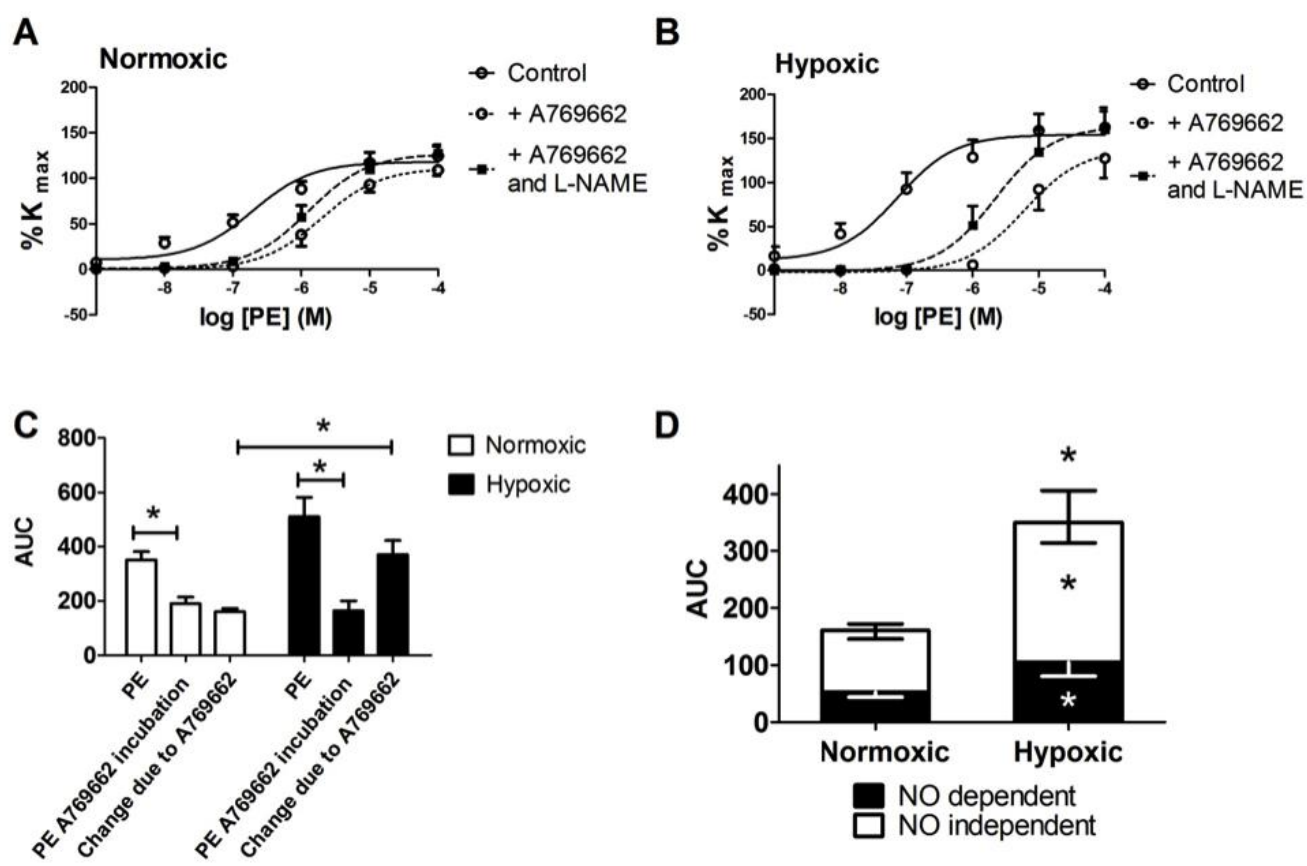


Figure 4

**Table 1.** Characteristics of the murine and human subjects.

Variable		Normoxic group	Hypoxic group	P value
<u>Mice:</u>				
Sample size		8	7-9	
Maternal body weight, gm	day 1	26.4 ± 1.8	26.2 ± 0.8	0.89
	day 14	31.9 ± 2.2	31.8 ± 0.8	0.93
	day 19	37.4 ± 3.0	32.5 ± 1.1	<b>0.046</b>
Litter size, n		6.0 ± 1.1	6.6 ± 0.4	0.59
Reabsorptions, n		0.4 ± 0.2	1.2 ± 0.4	0.06
Fetal weight, mg		1101.6 ± 11.0	816.2 ± 36.3	<b>&lt;0.0001</b>
Placental weight, mg		101.4 ± 7.5	95.5 ± 3.8	0.42
Fetal crown-rump length, mm		18.2 ± 0.3	16.3 ± 0.4	<b>0.002</b>
Fetal biparietal diameter, mm		5.4 ± 0.2	4.8 ± 0.1	0.03
Fetal biparietal diameter/weight		0.49 ± 0.02	0.58 ± 0.02	<b>0.006</b>
UtA internal circumference, □m		847.6 ± 34.1	911.8 ± 39	0.21
KCl maximum contraction, □m		1.08 ± 0.2	0.79 ± 0.1	0.20
PE maximal contraction, □m		124.8 ± 11.8	166.2 ± 17.5	0.07
	PD <sub>2</sub> , M	6.8 ± 0.29	7.1 ± 0.3	0.50
	PE+A769662 pD <sub>2</sub> without L-NAME, M	5.7 ± 0.17	5.2 ± 0.1	<b>0.045</b>
	PE+A769662 pD <sub>2</sub> with L-NAME, M	5.9 ± 0.16	5.6 ± 0.2	0.32
<u>Human subjects:</u>				
Sample size		3	3	
Placental weight, gm		670 ± 118	530 ± 35	0.33
Infant birth weight, gm		3813 ± 104	3263 ± 502	0.58
Infant gestational age, wk		39.2 ± 1.4	39.8 ± 1.7	0.78

Values are means ± sem. Bolded p values are <0.05.

Abbreviations: KCl = potassium chloride, L-NAME = N<sub>ω</sub>-Nitro-L-arginine methyl ester hydrochloride, PE = phenylephrine, pD<sub>2</sub> = negative logarithm to base 10 of the EC50 or the dose at which 50% of the maximal contraction was achieved.